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14. ABSTRACT We are completing this project to use a germ cell deficient Wv mouse model to test the hypothesis of a synergy between oncogenic mutations and postmenopausal biology in ovarian cancer development. We found that crossing of Wv mice into mutant p53 or pten (+/-) background did not lead to a malignant tumor phenotype (Aim 1). Instead, the mutants rescue ovarian germ cells, a very interesting finding. The ovarian surface epithelia in Wv/Wv: p27 (+/-) or Wv/Wv: p27 (+/-) compound mutant mice develop unique lesions with peculiar morphology and formed large ovarian tumors in older mice (Aim 2). The analysis of tumor phenotypes (Aim 3) is ongoing and we hope to complete and report the findings in next several months. Thus, in this project we have successfully developed Wv/Wv: p27 (+/-) mice as suitable models of ovarian epithelial cancer. We conclude that the result support our hypothesis that the collaboration of reproductive factors and genetic mutations leads to the development of ovarian cancer. The study also provides us with future directions, and we plan to seek future support to use flox-p53 mutant mice to create additional models. In sum, the project is completed as planned and is successful, and provides basis for further advance.					
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1. Introduction

This project is to use a unique Wv mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. Ovarian cancer often develops in women of peri-menopausal age, when ovulation ceases but gonadotropin levels are increased. We found that the germ cell deficient Wv mice mimics postmenopausal biology and develop benign ovarian tumors. We plan to test the hypothesis that the synergy between oncogenic mutations and postmenopausal biology can be revealed by combining the germ-cell deficient phenotype of the Wv/Wv mice and genetic alterations such as p53, pten, or p27kip1, which are found in human ovarian cancer.

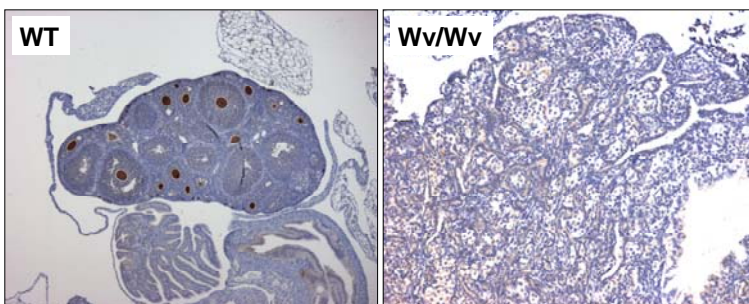
2. Body: Research Progress

In the first year of the project, we completed **Aim 1/Task 1.**, to determine the optimal genetic changes that synergize with the Wv genotype for the development of malignant ovarian tumors in mice (Months 1-12). We carried out studies to use the Wv mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. We tested the hypothesis that the synergy between oncogenic mutations and postmenopausal biology can be revealed by combining the germ-cell deficient phenotype of the Wv/Wv mice and genetic alterations such as p53, pten, or p27kip1, which are found in human ovarian cancer. We crossed and obtained several female Wv mice with additional p53 (-/-), p27 (-/-), or pten (+/-) mutation and analyzed the ovaries for tumor phenotype. The details of the results are described below for each combination of genotypes tested.

p53 deletion reverts ovarian tumor phenotype in Wv/Wv mice

At 3 months of age, the wildtype ovary contains many germ cells and follicles that are indicated by positive staining with marker PGC7 (**Figure 1**). The Wv/Wv ovary shows infiltration with epithelial derived tumor cells and contains no germ cells or follicles (**Figure 1**).

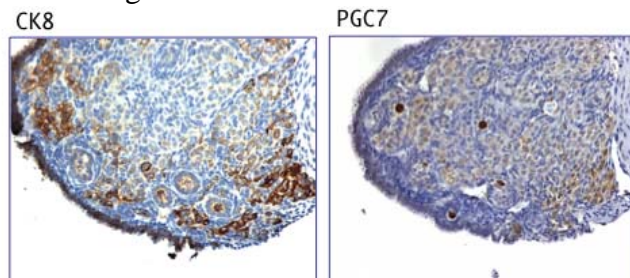
Figure 1. Staining of ovarian germ cells and follicles with PGC7 in wildtype and mutant mice:



Representative immunostaining of follicles with germ cell marker PGC7 in ovaries from 3-month-old wildtype and Wv/Wv. The ovaries are representative of 5 mice analyzed in the pilot study of the **Aim 1**.

We found that crossing Wv mice into mutant p53 did not lead to development of large tumors or a malignant tumor phenotype. Instead, the additional p53 mutation rescues ovarian germ cells, a very interesting finding. As shown in a representative figure (**Figure 2**), an ovary of Wv/Wv:p53 (-/-) mouse showed the absence of CK-8-positive epithelial tumor cells and the presence of PGC7-positive follicles (**Figure 2**). Thus, deletion of p53 in the Wv/Wv:p53 (-/-) ovary results in the absence of tumor and the presence of germ cells/follicles. This finding suggests p53 is highly important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key causal factor for the ovarian tumor phenotype.

Figure 2. Ovarian morphology in Wv/Wv:p53 (-/-) ovaries. An immunostaining with epithelial cell marker cytokeratin 8 (CK8) and germ cell marker PGC7 of a representative ovary of the 3-month-old Wv/Wv:p53 (-/-) mice. The staining shows that there is no tumor lesion in the ovary. The presence of several germ cells or follicles are indicated by the positive staining of PGC7 in an adjacent section of the same ovary. This ovary is representative of ovarian tissues harvested from 5 mice analyzed in the pilot study.



In future study, we plan to create a p53 mutation only in ovarian surface epithelial cells to avoid the rescuing/cell surviving activity in germ cells by a cre-lox conditional gene targeting approach. Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells.

Reduction of pten gene dosage has subtle influence on Wv/Wv ovarian tumor phenotypes

The addition of pten mutation into Wv/Wv mice did not significantly alter the tumor phenotype (**Figure 3**), rather it reduced the presence of epithelial tumor lesions in about one third of the ovaries. Interestingly, follicle structure was observed in the ovaries with a reduced tumor phenotype, similar to the addition of p53 mutation. It is possible that a reduction of pten gene dosage leads to an increased survival of ovarian follicles and germ cells, and thus a reduced tumor phenotype. We conclude that we need to introduce the additional mutations only to the surface epithelial cells but not to the germ cells of the Wv ovaries.

Figure 3. Ovarian morphological features of Wv/Wv:pten (+/-) mutant mice: Representative cytokeratin staining (of epithelial cells) of three ovaries from 3-month-old Wv/Wv:pten (+/-) mice. One ovary shown (right panel) exhibits a reduced tumor lesion. These ovaries are representative of ovarian tissues harvested from 5 mice each analyzed in the pilot study of the **Aim 1**.



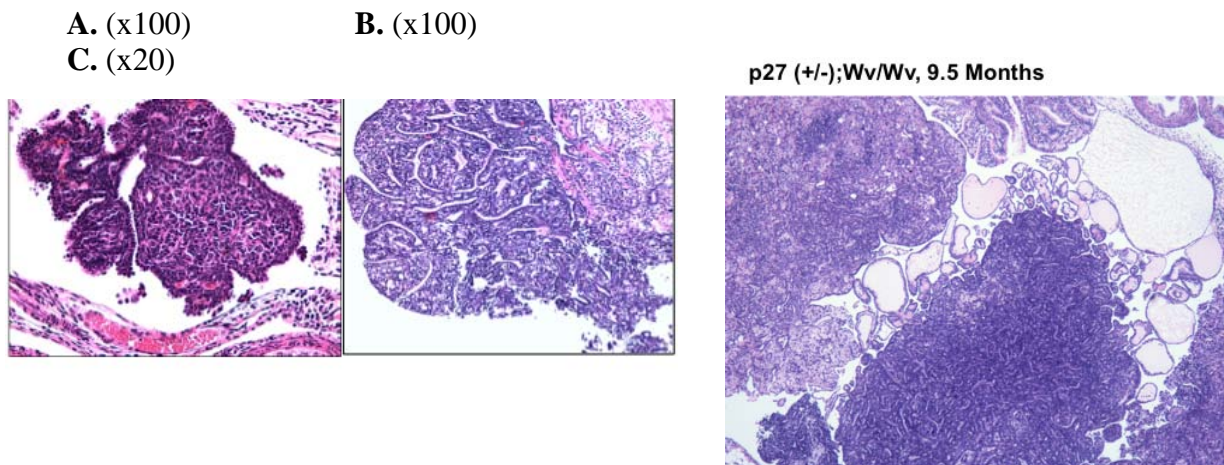
In future experiments, we should use Wv/Wv:pten (flox/flox) model to study pten deletion only in epithelial cells but not in follicles and germ cells.

p27kip1 deletion enhances Wv/Wv ovarian tumor phenotypes

Reduction of p27 expression predicts ovarian prognosis in human ovarian cancer, and p27 loss is likely important for ovarian cancer development. The addition of p27 deletion into Wv/Wv mice enhanced the tumor phenotypes. The ovarian surface epithelia in these compound mutant mice, both Wv/Wv:p27 (-/-) and Wv/Wv:p27 (+/-), develop lesions with unique morphology (**Figure 4A,B**), resembling papillo endometrial and serous carcinoma morphology. The addition of p27 (-/-) background often led to lymphoma phenotype in 6-month or older mice. In aged Wv/Wv:p27 (+/-) mice, large ovarian tumors had developed, invaded and embedded in the entire region (**Figure 4C**), often causing lethality of the tumor-bearing mice.

Thus, the Wv/Wv:p27 (+/-) ovarian tumor mouse model appears highly promising and it is one of our successes in **Aim 1**. Thus, we used Wv/Wv:p27 (+/-) ovarian tumor model in the more detailed analysis in **Aim 2**. Currently, we are continuing the analysis of Wv/Wv:p27 (+/-) mouse ovarian epithelial tumors for proliferation and expression of proteases, etc. The results will be ready for preparation in report shortly.

Figure 4. Ovarian morphology in Wv/Wv:p27 (-/-) and Wv/Wv:p27 (+/-) ovaries. H&E images of a representative ovary of the 4-month-old Wv/Wv:p27 (-/-) (**A**) and Wv/Wv:p27 (+/-) (**B**) mice. H&E staining shows the unique morphology of the ovarian tumor. By 9 months of age, ovarian tumors from Wv/Wv:p27 (+/-) are much enlarged and embedded in the entire ovarian/uterine region, highly resembling human ovarian cancer (**C**).



Summary and conclusions of Aim 1.

We completed all the experiments in **Aim 1**, and we reached a conclusion that Wv/Wv:p53 (-/-) and Wv/Wv:pten (+/-) mice do not develop neoplastic ovarian tumors, due to the rescuing of ovarian germ cells and follicles in the compound mutant mice.

We found Wv/Wv:p27 (-/-) and Wv/Wv:p27 (+/-) mice develop more malignant and bigger ovarian tumors, and will be proper mouse models for continue studying. Currently, we are completing the analysis ovarian tumor phenotypes from of these p27 mutant mice (**Aim 3**). The results will be ready for report in a few months.

In additional future study, we created mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells by a cre-lox conditional gene targeting approach (**Aim 2**). We crossed Wv mice into flox-p53 mutant mice. Then, cre recombinase was uinroduced into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This approach was considered in the original application as an alternative approach. Thus, *Task 2* (**Aim 2**) has been carried as planned. The experiments were carried out essentially as that were planned in the original application. The timeline and tasks in the Statement of Work have been well followed.

Adenoviral delivery of cre to ovarian surface epithelial cells

As part of **Aim 2**, we first established the approach for expressing cre in ovaries in the lab. When the mice are 2 and 6 months of age, the female Wv/+:p53 (flox/flox) mice will be injected into the ovarian bursa with Adv-cre to delete p53 and Adv-lacZ as controls in the ovarian surface epithelial cells. Currently, there is no suitable cre transgenic mouse line that can be used to specifically express in the ovarian surface epithelium. The protocol of adenoviral injection into ovarian bursa has been well established, and Adv-cre injection has been successfully used in several mouse models of ovarian tumors recently. Thus, we will use Adv-cre injection as a main approach in this aim. Adv-cre and Adv-lacZ were purchased commercially (Vector Lab, Iowa). Using Adv-lacZ as a reporter for transfection by Adenoviral injection, we observed expression of beta-galactosidase in cells of both the ovarian surface and the bursa inner layer of cells (**Figure 5**). We conclude that adenoviral delivery of cre into ovarian bursa is efficient and technically practical.

Figure 5. Delivery of Adenoviral lacZ to ovarian surface epithelial cells. Adv-lacZ was used as a reporter for adenoviral injection into ovarian bursa. The left ovary of a 2-month-old mouse was injected with 10^8 pfu of Adv-lacZ in 5 μ l, and the right ovary was injected with PBS as a control. Seven days after injection, ovaries were harvested, sectioned with a cryotome, stained for beta-galactosidase activity, and counterstained with Fast-Red. Area indicated by a “*” is shown in a higher magnification.



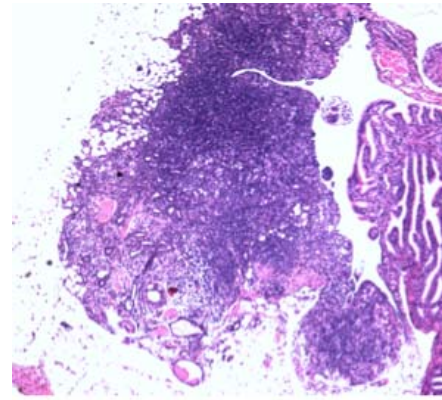
Ovarian tumor development in Wv/Wv:p53 (flox/flox)Adv-cre models

When the mice are 2 or 6 months of age, the Wv/Wv:p53 (flox/flox) female mice were injected with Adv-cre to delete p53 in the ovarian surface epithelial cells, and were sacrificed after 6-10 months to allow tumor development, until the mice are about 1 year of age.

By deleting p53 in ovarian surface epithelial cells through injection of adenovirus expression cre, we established another ovarian mouse model referred to as Wv/Wv:p53 (flox/flox):Adv-cre, mimicking both reproductive factors (postmenopause) and genetic mutation (p53) (**Figure 6**). These Wv/Wv:p53 (flox/flox):Adv-cre ovarian epithelial tumors appear malignant, though we will need more time and number of mice to reach a statistically significant conclusion..

We are currently characterizing in more details of these two mouse ovarian epithelial models. Thus, we have established two models of ovarian epithelial tumors, of Wv/Wv:p27 (-/-):Adv-cre and Wv/Wv:p53 (flox/flox):Adv-cre.

Figure 6. Ovarian tumor morphology in Wv/Wv:p53 (flox/flox);adv-cre mice. H&E images of representative ovarian tumor of the 6-month-old Wv/Wv:p53 (flox/flox) mouse. The mouse was injected with cre delivering adenovirus (Adv-cre) into ovarian bursa at 3 months of age, and the ovarian tumor was analyzed at 6 months of age.



Studies of human cancer for relevance

Task 3 is “To analyze the alterations in signaling pathways in ovarian tumors and derived cell cultures from the mice (Months 30-36)”. The work is ongoing, to further analyze the Wv/Wv:p27 (-/-):Adv-cre and Wv/Wv:p53 (flox/flox):Adv-cre models.

To collaborate with the mouse model study, we have also examined human ovaries obtained from prophylactic oophorectomies for morphological changes as what we attempt to model using the Wv mice. We assembled a panel of archived ovarian tissues: 52 ovarian tissue blocks were from prophylactic oophorectomies of a high-risk (BRCA1/2 mutation or a family history of breast or ovarian cancer) population; 66 ovaries were from surgeries due to non-ovarian-related diseases, referred to as normal-risk group. The morphology of ovarian tissues was examined, and morphological changes including papillomatosis, invaginations, inclusion cysts, and epithelial stratification were assessed in a blinded fashion. We found that inclusion cysts and deep invaginations were found much more commonly in women age 45–54 of either high risk or normal risk groups. When age was categorized into two groups, 45-54 representing peri-menopausal status and the other group consisting of the remaining age groups (below 45 years and 55 years & above), a statistically significant difference was found between age group and frequency of occurrence of morphological features. The odds of occurrence of inclusion cyst were 5.43 times as high in women aged 45-54 relative to other women (p-value = 0.009). Likewise, the odds of occurrence of deep invagination were 6.42 times as high in women aged 45-54 relative to other women (p-value = 0.008), and the odds of occurrence of Pseudo-

stratification were 3.77 times as high in this group of women as in other women (p-value = 0.039). This study suggests that the frequency of these histological features, especially inclusion cysts, may associate with age or menopausal status. We propose that ovulatory and perimenopausal gonadotropin stimulation produces ovarian morphological changes, and these histological features may promote the transformation of genetically compromised epithelial cells in the development of ovarian cancer. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.

Therefore, we report that the project has been progressed well as planned in the last 3 years. We found that the additional mutations had more impact on the germ cells than the epithelial cells. We have also ayeared the basis for future studies of the models.

Conclusions:

We present observations suggesting that the ovarian surface epithelial cells and derived inclusion cysts are sources of cancer cells, at least in a fraction of ovarian cancer. Our studies of the Wv mouse ovarian tumor phenotypes suggest that Wv mice are suitable to model ovarian aging and the impact of reproductive factors (such as follicle depletion and menopause) on ovarian cancer risk.

The enhanced ovarian tumor phenotype of Wv/Wv and p27kip1 deleted mice is a proof-of-principle for our proposal to increase the malignancy of Wv ovarian tumor by adding genetic mutation(s) to the prior benign ovarian epithelial tumors. Currently, Wv/Wv:p27 (+/-) mice are useful ovarian tumor mouse models that will allow us to carry out experiments before additional number of the potentially superior models (possibly Wv/Wv:p53 (flox/flox):Adv-Cre) are produced and characterized.

Preliminary studies with p53 and pten deletion confirm the causative mechanism of follicle depletion in the development of Wv ovarian epithelial tumors. The results also indicate that we need to delete p53 specifically in ovarian surface epithelial cells but not in germ cells to create a model of malignant ovarian tumor based on the Wv mice.

In the future study, we plan to create mutations only in ovarian surface epithelial cells to avoid the rescuing activity of p53 deletion for follicles and germ cells. Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells.

3. Key Research Accomplishments

- (1) Further verify the relevance of the Wv mouse model to human menopausal biology.
- (2) Verifying the influence of reproductive aging/menopause on human ovarian morphological changes. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.
- (3) We made an unexpected but very interesting finding that the additional mutation of p53 in the Wv mice rescued ovarian tumor phenotype and preserved germ cells. This finding suggests p53 is very important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key for the ovarian tumor phenotype.
- (4) We established a suitable ovarian cancer mouse model by combining reproductive factor (follicle depletion in Wv mice) and p27 suppression of p27 knockout background. The Wv/Wv:p27 (+/-) mice develop large ovarian tumors. Thus, we provide additional support for our hypothesis that the collaboration of reproductive factors and genetic mutations leads to the development of ovarian cancer.
- (5) Based on the new information learned from the ongoing study, we consider to make oncogenic mutation (such as deletion of p53 or pten) in only ovarian surface epithelial cells but in germ cells/follicles. We did preliminary study to establish the delivery of cre using adenoviral vector (Adv-cre) into ovarian surface epithelial cells. In a very limited number of mice tested, we are able to produce malignant ovarian tumors by conditionally deleting p53, in a Wv/Wv:p53 (flox/flox):Adv-Cre model. This result provides basis for a future project, and we will seek additional funding to support the experiments.

4. Reportable Outcomes

- (1) Yang et al., Am. J. Pathology, 2007: We showed that the Wv mice are excellent models for human menopausal biology. This result is included in a paper (Yang et al, 2007).
- (2) Cai et al., Gyn. Oncology 2006: We found that the human ovaries show age-dependent morphological changes, suggesting follicle depletion and gonadotropin stimulation in peri-menopausal period resulting in ovarian morphological changes. This observation has been published (Cai et al, 2006).
- (3) Cai, Yang, Smith et al., 2008 in preparation: We found that p53, pten, and p27kip1 genes have critical impacts on the survival of ovarian germ cells/follicle. We are doing additional experiments to characterize the mechanism further and

also to obtain additional cases to determine the statistical significance. These results will be prepared for publication in 2-3 months.

- (4) Smith et al., 2008 in preparation: We have established the mouse models of ovarian cancer in Wv/Wv:p27 (+/-) and Wv/Wv:p53 (flox/flox):Adv-cre mice. Although it is clear that these mouse models will be highly valuable to study ovarian cancer etiology and biology, we will need additional characterization and a larger number of mice to be analyzed in order to reach statistically significant conclusions. We may need to obtain additional funding support to complete these very interesting findings. We hope to publish these results in another 1-2 years.

5. Conclusions:

The experimental results are supportive of the hypothesis that. We need to delete p53 specifically in ovarian surface epithelial cells but not in germ cells in order to create a model of malignant ovarian tumor based on the Wv mice.

In the future study, we plan to create mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells. Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This approach was considered in the original application as an alternative approach. Thus, the future experiments will be carried out essentially as that were planned in the original application.

In sum, the project is progress well as planned. We have layered the basis in the first year of the project, and we hope to obtain conclusive results for the aims and questions proposed in the coming years.

6. References

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7. Appendices

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